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Cytoprotective effect of the small GTPase RhoB expressed upon treatment of fibroblasts with the Ras-glucosylating *Clostridium sordellii* lethal toxin

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ABSTRACT

Mono-glucosylation of (H/K/N)Ras by *Clostridium sordellii* lethal toxin (TcsL) blocks critical survival signaling pathways, resulting in apoptosis. In this study, TcsL and K-Ras knock-down by siRNA are presented to result in expression of the cell death-regulating small GTPase RhoB. TcsL-induced RhoB expression is based on transcriptional activation involving $p38_{alpha}$ MAP kinase. Newly synthesized RhoB protein is rapidly degraded in a proteasome- and a caspase-dependent manner, providing first evidence for caspase-dependent degradation of a Rho family protein. Although often characterised as a pro-apoptotic protein, RhoB suppresses caspase-3 activation in TcsL-treated fibroblasts. The finding on the cytoprotective activity of RhoB in TcsL-treated cells re-enforces the concept that RhoB exhibits cytoprotective rather than pro-apoptotic activity in a cellular background of inactive Ras.

1. Introduction

The low molecular mass GTP binding proteins RhoA, RhoB and RhoC act as molecular switches, as they cycle between an active GTP-bound conformation and an inactive GDP-bound conformation. Rho-GTPases regulate the actin dynamics, cell adhesion, cell cycle progression, gene transcription, intracellular membrane trafficking, and cell survival [1]. These functions are important in tumorigenesis and tumor progression [2]. RhoA and RhoC are over-expressed in various types of tumors, and over-expression of RhoC protein has been shown to correlate with tumor metastasis [3,2]. The role of RhoB in human cancer development is controversial. RhoB has been found to be down-regulated in head and neck carcinoma and gastric and lung cancer but overexpressed in breast cancer [4,2]. Due to its pro-apoptotic activity RhoB is generally regarded as a tumor suppressor [5]. RhoB expression significantly inhibits proliferation, migration and invasion of cancer cells and also enhances chemosensitivity of malignant cells to anticancer drugs [6–9].

RhoB is an immediate-early gene product with a short half life period. RhoB expression has been reported in response to extracellular stimuli such as growth factors, cytotoxic and genotoxic agents [10–12]. The low intracellular level of RhoB is maintained by repression of *rhoB* promoter activity by oncogenes, including the epidermal growth factor receptor (EGFR), ErbB2, and Ras [8,9]. Farnesyltransferase inhibitors (FTIs) inhibits Ras through prevention of posttranslational farnesylation. FTI-induced Ras inhibition results in RhoB expression [13]. RhoB has been identified as a target in cancer therapy, based on the concept that RhoB expression is required for apoptosis of transformed cells [5,14].

Lethal Toxin from *Clostridium sordellii* (TcsL) is the major virulence factor of *C. sordellii*-associated disease in animals and humans, which includes myonecrosis, sepsis and shock [15,16]. TcsL enters target cells by receptor-mediated endocytosis and efficaciously mono-glucosylates (and thereby inactivates) the Ras subtype proteins (H/K/N)Ras and Rap(1/2). TcsL less efficaciously glucosylates Rac1 and Cdc42, while Rho(A/B/C) are not glucosylated [17–19]. Treatment of cells with TcsL results in inhibition of PI3K/

Abbreviations: FTI, farnesyltransferase inhibitor; MEF, mouse embryonic fibroblasts; TcdBF, toxin B from the *Clostridium difficile* serotype F strain 1470; TcsL, lethal Toxin from *Clostridium sordellii*; TUDCA, tauroursodesoxycholic acid

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Akt-mediated survival signaling and activation of p38 MAP kinase, with both effects contributing to the initiation of apoptosis of cultured myeloid and epithelial cells [20–23].

In this study, TcsL-induced RhoB expression is characterised in detail. TcsL treatment is presented to result in pronounced RhoB expression based on transcriptional activation involving $p38_{alpha}$ MAP kinase. RhoB, that is not glucosylated by TcsL, becomes activated in TcsL-treated cells and protects cells from TcsL-induced apoptosis. RhoB is caspase-dependently degraded, which is interpreted in terms of an amplification of pro-apoptotic signaling process initiated by the toxin. This is the first report showing that a Rho subtype protein is degraded in a caspase-dependent manner.

2. Materials and methods

2.1. Materials

Toxin B (TcdBF) and TcsL were purified from the *Clostridium difficile* serotype F strain 1470 and the *Clostridium sordellii* strain 6018, respectively [24]. The GST-C21 vector construct was a kind gift of Dr. John Collard (Amsterdam). Commercially obtained reagents: Actinomycin D (Calbiochem), cycloheximide (Calbiochem), caspase inhibitor I. (Z-VAD(OMe)-fmk) (Calbiochem), LY294002 (Calbiochem), MG132 (Calbiochem), SB203580 (Calbiochem), Tauroursodeoxycholic acid (Sigma); antibodies: K-Ras(Mab F234, SantaCruz); Ras(Mab27H5, Cell Signaling); Rac1(Mab 102; Transduction Lab); Rac1(Mab 23A8, Upstate); RhoA(Mab 26C4, SantaCruz); RhoB (Mab C-5, SantaCruz); beta-actin(Mab AC-40, Sigma), and horseradish peroxidase conjugated secondary antibodies rabbit/mouse (Rockland).

2.2. Methods

2.2.1. Cell culture

NIH3T3 fibroblasts, E1A/Ras-transformed RhoB +/− and RhoB −/− mouse embryonic fibroblasts (MEFs) (kindly provided by Dr. George Prendergast, Lankenau Institute for Medical Research, Wynnewood, Pennsylvania) [7,25], and $p38_{alpha}$−/− and the corresponding $p38_{alpha}$ wt MEFs (kindly provided by Dr. Angel Nebreda, Institute for Research in Biomedicine, Barcelona, Spain) [26] were cultivated in Dulbecco's modified essential medium (Biochrom + 10% FCS, 100 μg/ml penicillin, 100 U/ml streptomycin and 1 mM sodium pyruvate) at 37 °C and 5% CO_2 according to standard protocols. For all experiments, cells were seeded subconfluently into 3.5 cm-diameter dishes. Cells were washed once with phosphate buffered saline. They were then scraped into Laemmli sample buffer (200 μl). The obtained suspension was incubated for 10 min at 37 °C and 1400 rpm in a thermo shaker and subsequently sonified on ice. The lysate was then incubated for 10 min at 95 °C and submitted to Western blot analysis.

2.2.2. RhoB activity assay

The Rho-binding domain of Rhotekin, C21, was expressed as GST-fusion protein in *Escherichia coli*. After their lysis using French Press, the soluble fraction was obtained by centrifugation (20.000 rpm, 20′). It was incubated with glutathione–Sepharose for 30′ at 4 °C and subsequently washed. 3T3 fibroblasts treated with either TcdBF or TcsL as indicated were lysed in lysis buffer (50 mM Tris pH 7.2, 150 mM NaCl, 5 mM $MgCl_2$, 1% NP-40, 1 mM PMSF, 5 mM DTT, Complete –EDTA). The soluble fraction was obtained by centrifugation (10000×*g*, 5′). It was then added to the glutathione-bound GST-C21 for 1 h (4 °C). After washing of the beads, RhoB was eluted by incubation with Laemmli sample buffer at 95 °C (10′). Samples were submitted to SDS–PAGE and Western blotting.

2.2.3. Knockdown of gene expression using siRNAs

The following Ras-specific siRNAs were used in this study: siRNA to H-Ras, GAGUGGAGGAUGCCUUCUA; siRNA to K-Ras, GCUC AGGACUUAGCAAGAA; and siRNA to N-Ras, AAAGCGCACUGA CAAUCCA. These siRNAs (20 nM) were transfected into Hela cells (n = 50,000) by using JetPRIME transfection reagent (PeqLab, Erlangen, Germany) according to the manufacturer's protocol. After 48 h, the effects of knockdown on RhoB expression were assessed. The level of protein knockdown by siRNA was determined by Western-blot analysis.

2.2.4. RNA purification and RT-reaction

Total RNA was purified from fibroblasts using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Shortly, cells were lysed in lysis buffer. The RNA was extracted and bound to silica membranes after addition of ethanol. Contaminating DNA was cleaved by DNase I digestion and bound RNA washed with the supplied buffer. Total cellular RNA was then eluted with RNase free water. 2 μg of RNA were then used as template in the RT-reaction, which was performed with the Omniscript RT-Kit (Qiagen) according to the manufacturer's instructions.

2.2.5. Semi-quantitative real-time PCR

The real-time PCR was conducted using the QuantiTect SYBR Green PCR Kit (Qiagen) and a LightCycler (Roche). The cDNA obtained from the RT-reaction was diluted (1:1000) to avoid overloading. Primers (3 μM) used were: β-actin: 5′-CCT GCT TGC TGA TCC ACA TC-3′ and 5′-GCA TTG CTG ACA GGA TGC AG-3′, RhoB: 5′-CCG AGG TAA AGC ACT TCT GC-3′ and 5′-CCG AGC ACT CGA GGT AGT CA-3′. After every run, a melting curve was recorded to ensure the specificity of the reaction.

2.2.6. Analysis of rhoB promoter activity

Subconfluent NIH3T3 fibroblasts were transiently transfected with 2 μg of the 3.5 kb *rhoB* promoter CAT construct [10] for 14 h, applying FuGENE 6 Transfection Reagent (Roche). Cells were then treated as indicated and harvested. The protein concentration of the lysates was normalized using Bradford test. The level of CAT expression was analyzed using an enzyme-linked immunosorbent assay (CAT-Elisa kit, Roche) according to the manufacturer's instructions.

2.2.7. Caspase-3 colorimetric assay

Caspase-3 activity was analyzed using the Caspase-3 colorimetric assay (R&D Systems), according to the manufacturer's instructions. Cells were exposed to the toxins and drugs as indicated. Cells were lysed and subsequently incubated with the colorimetric substrate DEVD-pNA at 37 °C for 3 h. The caspase-3-dependent release of the reporter molecule pNA was quantified by photometry using a scanning multi-well spectrophotometer at 405 nm.

3. Results

3.1. TcsL-induced expression and activation of RhoB

Pronounced RhoB expression was observed upon treatment of NIH3T3 fibroblasts with increasing concentrations of TcsL (Fig. 1A). TcsL-catalysed glucosylation of (H/K/N)Ras and Rac1 was evidenced using the glucosylation-sensitive Ras(27H5) and Rac1(102) antibodies, respectively [27,17]. Either antibody recognizes glucosylation in terms of apparently decreasing levels of either (H/K/N)Ras or Rac1 protein (Fig. 1A). (H/K/N)Ras was more efficaciously glucosylated than Rac1 (Fig. 1A), consistent with former observations [17]. The levels of K-Ras or Rac1 were not changed upon TcsL treatment, as analyzed using alternative

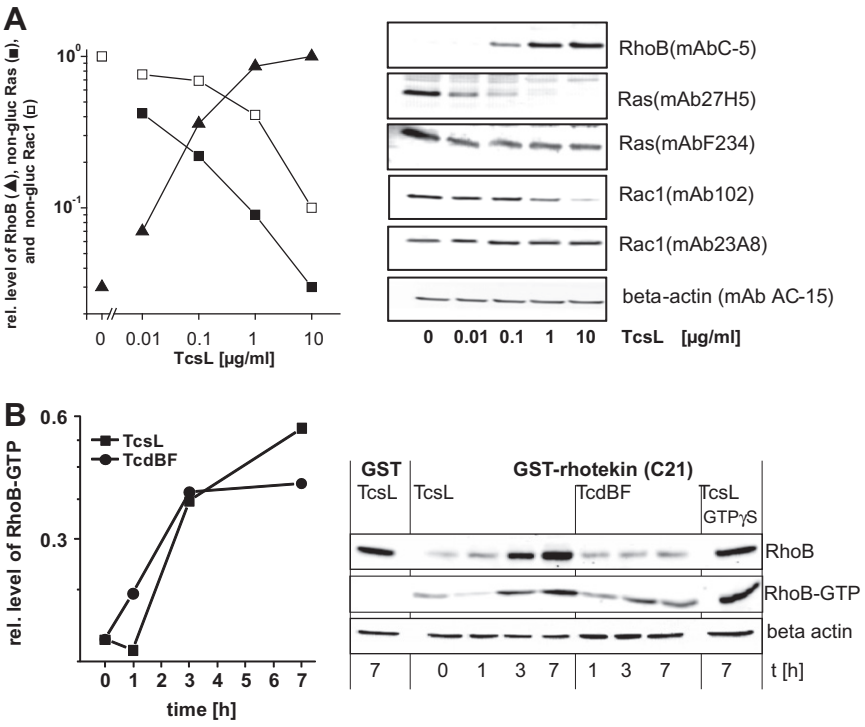

Fig. 1. RhoB expression and activation in TcsL-treated cells. (A) Correlation of Ras glucosylation and RhoB expression. NIH3T3 fibroblasts were exposed to the indicated concentrations of TcsL for 4 h. Cells were lysed and submitted to immuno-blot analysis using the indicated antibodies. Immuno-blots from representative experiments are presented ($n = 3$). Signal intensities obtained from immuno-blots applying the glucosylation-sensitive Ras(27H5) antibody (filled squares), the glucosylation-sensitive Rac1(102) antibody (open squares), and the RhoB antibody (filled triangles) from TcsL-treated cells were quantified and normalized to beta-actin. The concentration of either (H/K/N)Ras, Rac1, or RhoB from non-treated cells was set 1.0. Results displayed are the mean of three independent experiments. (B) Difference in RhoB expression between TcsL and TcdBF. Fibroblasts were exposed to TcsL (3 µg/ml) or TcdBF (30 ng/ml) for the indicated times. Cells were then lysed and analysed for the cellular level of RhoB-GTP using GST-rhotekin-C21 pulldown assay. GST alone served as a negative control. Total and precipitated RhoB was detected by immuno-blot analysis. A sample treated with TcsL for 7 h subjected to nucleotide exchange with the non-hydrolysable GTPγS indicated the level of maximal level of active RhoB-GTPγS, which was set 1.0. Signal intensities of RhoB activation induced by either TcsL (filled squares) or TcdBF (filled triangles) were quantified, normalized, and were given relative to the maximal level of RhoB-GTP[S].

K-Ras(F234) and Rac1(23A8) antibodies. TcsL-induced RhoB expression was also characterized in a time-dependent experiment (Fig. 1B, S1). After about 7 h of TcsL treatment, the RhoB level was elevated by about 10-fold (compared to the RhoB level in non-treated cells) and reached a steady state level (Fig. S1). Elevated RhoB level persisted for at least 17 h (Fig. S1). TcsL-induced RhoB expression was accompanied by a higher level of RhoB-GTP (Fig. 1B), as analysed by Rhotekin pull-down assay. RhoB, that is not glucosylated by TcsL, was thus activated in TcsL-treated cells. Taken together, TcsL induced RhoB expression and RhoB activation.

3.2. Critical role of (H/K/N)Ras inhibition in TcsL-induced RhoB expression

To provide evidence that (H/K/N)Ras glucosylation was essential for TcsL-induced RhoB expression, we exploited the related Rac-glucosylating variant toxin B from the *C. difficile* serotype F strain 1470 (TcdBF). TcdBF glucosylates specifically Rac1 but not (H/K/N)Ras [17,28]. TcsL and TcdBF were applied in equipotent concentrations with respect to Rac1 glucosylation (Fig. S2). TcsL, but not TcdBF, glucosylated (H/K/N)Ras and caused a decrease of the level of the phosphorylated form of the Ras downstream target p44/42 MAP kinase (ERK) (Fig. S2). TcsL (but not TcdBF) stimulated RhoB expression (Fig. 1B). This observation strongly suggested that (H/K/N)Ras glucosylation (rather than Rac1 glucosylation) is required for RhoB expression.

TcdBF treatment resulted in RhoB activation, although it did not induce RhoB protein expression (Fig. 1B). To further show that RhoB expression was based on Ras inhibition, RhoB expression was analysed in HeLa cells with (H/K/N)Ras knocked-down by a siRNA based method (Fig. 2A). Efficient knock-down of (H/K/N)Ras was confirmed using the (H/K/N)Ras-specific antibody Ras(mAb27H5) (Fig. 2A) [17]. (H/K/N)Ras knock-down induced RhoB expression (Fig. 2A), showing that (H/K/N)Ras inhibition is sufficient to stimulate RhoB expression. (H/K/N)Ras knock-down-induced RhoB expression was less efficaciously compared to RhoB expression stimulated by (H/K/N)Ras glucosylation. This difference correlated with the observation that TcsL completely inhibited (H/K/N)Ras, while traces of (H/K/N)Ras were still detectable upon (H/K/N)Ras knock-down (Fig. 2A). The low amount of residual (H/K/N)Ras were in fact responsible for reduced RhoB expression in cells with (H/K/N)Ras knocked-down, as TcsL treatment of these cells resulted in an increase in RhoB expression to an extent comparable to that observed in TcsL-treated cells (Fig. 2A). These observations showed that inhibition of Ras by either depletion or glucosylation resulted in RhoB expression. The observation that TcsL induced RhoB expression to comparable extent in cells with and without (H/K/N)Ras knocked-down further showed that glucosylation of Ras resulted in mere Ras inhibition but not in a gain of dominant-negative function of Ras. TcdBF treatment of cells with (H/K/N)Ras knocked down resulted in partial Rac1 glucosylation but did neither reduced the level of active Ras nor augmented RhoB expression (Fig. 2A). These observations excluded that Rac1 glucosylation triggered RhoB expression.

Next, the Ras isoform involved in RhoB expression was identified by knock-down of individual Ras isoforms in Hela cells. RhoB expression was specifically observed upon knock-down of K-Ras (neither of H-Ras nor N-Ras) (Fig. 2B). K-Ras knock-down was

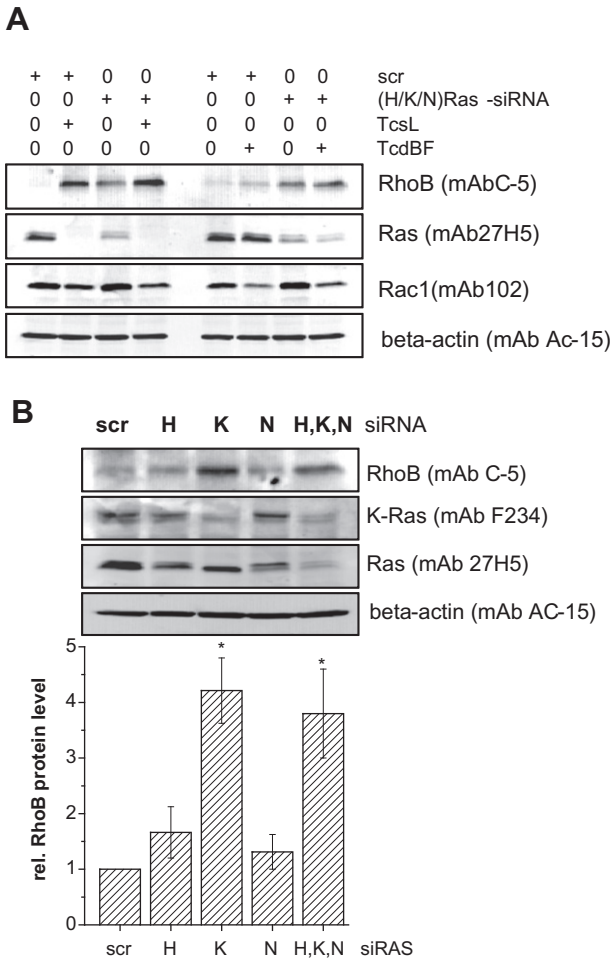

Fig. 2. RhoB expression upon Ras knock-down. (A) HeLa cells were transfected with siRNAs targeting H-Ras, K-Ras and N-Ras or with scrambled control siRNA (scr) as indicated for 48 h. Cells were subsequently treated with TcsL (5 μg/ml) or buffer for 4 h. The cellular levels of Ras and RhoB were analyzed by immuno-blot analysis. Signal intensities obtained from RhoB immuno-blots were quantified and normalized to beta-actin. Results displayed are the mean ± S.D. of three independent experiments. A *P*-value < 0.05 (*) was considered as statistically significant. (B) Ras depletion-induced RhoB expression. HeLa cells exposed to H-Ras-, K-Ras, or N-Ras-specific siRNA or scrambled (scr) siRNA as indicated for 48 h. Cells were lysed and submitted to immuno-blot analysis using the indicated antibodies. Immuno-blots from representative experiments are presented. Signal intensities obtained from immuno-blots applying the RhoB antibody were quantified and normalized to beta-actin. The RhoB level from non-treated cells was set 1.0. Results displayed are the mean ± S.D. of three independent experiments. A *P*-value < 0.05 (*) was considered as statistically significant as compared with non-treated cells.

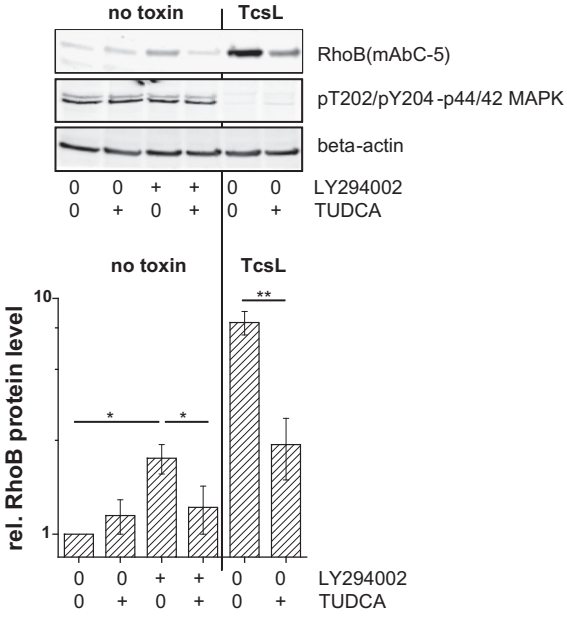

Fig. 3. Critical role of PI3K in TcsL-induced RhoB expression. NIH3T3 fibroblasts were treated with TUDCA (300 μM), Ly294002 (20 μM), and TcsL (3 μg/ml) as indicated for 4 h. Signal intensities were densitometrically quantified and normalized to the β-actin signal. The RhoB level from non-treated cells was set to 1.0. Data shown represent the mean of three independent experiments. *P*-values < 0.05 (*) and < 0.01 (**) were considered as statistically significant.

confirmed by strongly reduced K-Ras protein level as detected by the use of the K-Ras-specific antibody K-Ras(mAbF234) (Fig. 2B). Knock-down of either H-Ras and N-Ras was shown by reduced Ras proteins levels detection using (H/K/N)Ras-specific antibody Ras(mAb27H5) (Fig. 2B) [17]. K-Ras depletion was thus sufficient for RhoB expression. Apparently, specifically K-Ras (not H-Ras or N-Ras) repressed RhoB expression in Hela cells.

3.3. Involvement of PI3K in Ras-dependent RhoB repression

Ras-dependent repression of RhoB has been suggested to involve the Ras downstream effector PI3K [8,9]. Consistent with this proposal, treatment of fibroblasts with LY294002, an inhibitor of PI3K, resulted in a moderate RhoB expression (Fig. 3). The hydrophilic bile acid tauroursodesoxycholic acid (TUDCA), an activator of PI3K, has recently been reported to preserve PI3K activity downstream of glucosylated Ras [20,21,29]. Therefore, we hypothesized that TUDCA-induced activation of PI3K maintains RhoB repression in TcsL- or LY294002-treated cells. TcsL-induced and LY294002-induced RhoB expression was significantly reduced, if fibroblasts were additionally treated with TUDCA (Fig. 3). This observation suggested that the maintenance of PI3K activity downstream of glucosylated Ras preserved RhoB repression and prevented its expression in TcsL- or LY294002-treated cells. TUDCA treatment did not prevent TcsL-induced p44/42 MAPK dephosphorylation (Fig. 3), excluding that TUDCA interfered with TcsL-induced Ras inactivation and that TUDCA non-specifically activated Ras downstream kinases. TUDCA-mediated PI3K activation (downstream of glucosylated Ras) thus preserved RhoB repression in TcsL-treated cells.

3.4. Critical role of transcriptional activation in TcsL-induced RhoB expression

TcsL-induced RhoB expression was accompanied by a concentration-dependent expression of *rhoB*-mRNA, as analysed using real-time RT-PCR (Fig. 4A). TcsL-induced expression of either *rhoB*-mRNA (Fig. 4A) or RhoB protein (Fig. 4B) was prevented upon pre-treatment of cells with actinomycin D. Expression of RhoB proteins was also blocked upon pre-treatment of cells with cycloheximide (Fig. 4B). These findings strongly suggested that increased TcsL-induced RhoB expression rests on transcriptional activation. In fact, TcsL activated the *rhoB* promoter in a concentration dependent manner, as analysed using a 3.5 kb fragment of the *rhoB* promoter cloned into pCAT basic as a reporter construct [10] in NIH3T3 fibroblasts (Fig. 4C).

3.5. Involvement of $p38_{alpha}$ MAP kinase in TcsL-induced RhoB expression

Several reports suggested a role of p38 MAP kinase in the regulation of RhoB expression [30,31]. TcsL time-dependently activated

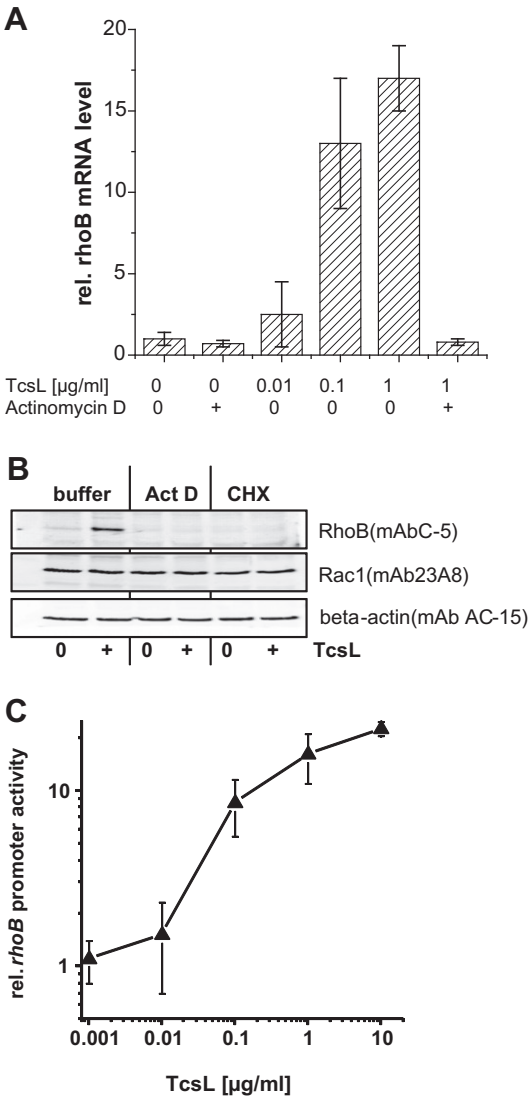

Fig. 4. Critical role of transcriptional activation in TcsL-induced RhoB expression. (A) TcsL-induced *rhoB*-mRNA expression. Fibroblasts were treated with the indicated concentrations of TcsL and actinomycin D (5 µM) as indicated for 4 h. Subsequently, total mRNA was prepared from treated cells and submitted to real-time RT-PCR. *rhoB* signals were normalized to *beta-actin* signals. The level of *rhoB*-mRNA in non-treated cells was set 1.0. Results displayed are the mean ± S.D. of three independent experiments. (B) Fibroblasts were treated with TcsL (3 µg/ml) or actinomycin D (Act D, 5 µM) or cyloheximide (CHX, 100 µM) as indicated for 4 h. Cellular RhoB levels were analysed by immuno-blot analysis. (C) TcsL-induced activation of the *rhoB* promoter. NIH3T3 fibroblasts were transfected with the 3.5 kb *rhoB* promoter CAT-construct followed by treatment of the cells with the indicated concentrations of TcsL for 7 h. Cells were harvested for analysis of CAT protein levels using ELISA. Data shown represent the mean (± S.D.) of three independent experiments.

p38 MAPK in fibroblasts, as analysed by measuring the increasing levels of pT180/pY182-p38 MAP kinase (Fig. S3). TcsL-induced Rac1 glucosylation correlated with p38 activation (Fig. S3). Murine fibroblasts specifically express $p38_{alpha}$ [32,26], for which reason the activities of the $p38_{beta/gamma/delta}$ do not have to be considered. To show that $p38_{alpha}$ is involved in TcsL-induced RhoB expression, RhoB expression was differentially analysed in SV40-immortalized $p38_{alpha}-/-$ and the corresponding $p38_{alpha}$wildtype MEFs [32,26]. The expression of RhoB protein was reduced in TcsL-treated $p38_{alpha}-/-$ MEFs compared to the corresponding $p38_{alpha}$wildtype MEFs (Fig. 5A), suggesting a role of p38 in the regulation of RhoB expression. TcsL glucosylated (H/K/N)Ras in both cell lines with comparable kinetics (Fig. 5A), excluding that reduced RhoB expression in $p38_{alpha}-/-$ MEFs was due to attenuated Ras glucosylation. The activation of the *rhoB* promoter was more pronounced in $p38_{alpha}$wildtype MEFs than in $p38_{alpha}-/-$ MEFs (Fig. 5B). Furthermore, TcsL induced expression of *rhoB*-mRNA in $p38_{alpha}$wildtype more efficaciously than in $p38_{alpha}-/-$ MEFs (Fig. S4). Upon inhibition of transcription by actionomycin D, cellular *rhoB*-mRNA levels decayed with first order kinetics in either cell line (Fig. S4). The half-life of *rhoB*-mRNA was estimated to 35 ± 4 min in $p38_{alpha}$-wildtype and to 38 ± 8 min in $p38_{alpha}-/-$ MEFs (Fig. S4). These observations most likely indicated that $p38_{alpha}$-mediated stabilisation of *rhoB*-mRNA does not contribute to TcsL-induced RhoB expression. SB203580 is a pyridinylimidazole inhibitor of the $p38_{alpha/beta}$ [33]. Pre-treatment of $p38_{alpha}$wildtype MEFs with SB203580 also resulted in reduced RhoB expression upon TcsL treatment (Fig. S5), consistent with the findings obtained above from $p38_{alpha}-/-$ and $p38_{alpha}$wildtype MEFs (Fig. 5A). In $p38_{alpha}-/-$ MEFs, the kinetics of TcsL-stimulated RhoB expression were comparable upon SB203580 treatment, arguing against an off-target effect of SB203580 (Fig. S5). The glucosylation of (H/K/N)Ras appeared to be moderately reduced in $p38_{alpha}-/-$ ME fibroblasts (Fig. S5), suggesting that SB203580 weakly interfered with TcsL-catalysed Ras glucosylation. Upon genetic deletion of $p38_{alpha}$ and pharmacological inhibition of $p38_{alpha}$ by SB203580, TcsL-induced activation of the *rhoB* promoter and RhoB expression was reduced, pointing to a role of $p38_{alpha}$ in regulating RhoB expression. The influence of $p38_{alpha}$ in RhoB expression appeared to be independent of stabilizing *rhoB*-mRNA.

3.6. Proteasome- and caspase-dependent degradation of RhoB

To check if increased stability of RhoB protein contributed to TcsL-induced RhoB expression, the half live of RhoB was analysed in fibroblasts upon TcsL treatment for 4 h. RhoB de novo synthesis was blocked by cycloheximide and the RhoB level rapidly decreased, with a half-life of about 50 min (Fig. 6). Rho proteins are well established to be subject of proteasome-dependent degradation [34,27]. Interestingly, RhoB degradation was only reduced (not fully prevented), when proteasomal degradation was blocked using the proteasome inhibitor MG132 (Fig. 6). In the presence of MG132, half-life of RhoB increased to about 3 h (Fig. 6). TcsL is well established to induce apoptotic cell death of cultured cells [20,35], leading to the hypothesis that caspase-dependent degradation of RhoB might influence the short RhoB half-life in TcsL-treated cells. In fact, RhoB degradation was slowed down, when fibroblasts were pre-treated with Z-VAD-fmk, a broad-spectrum inhibitor of caspases (Fig. 6). Combined pre-treatment of fibroblasts with MG132 and Z-VAD-fmk completely blocked RhoB degradation (Fig. 6). These findings show that in TcsL-treated fibroblasts RhoB was degraded in a proteasome- as well as caspase-dependent manner. The half-life of RhoB from TcsL-treated fibroblasts of <1 h is clearly below the reported half-live of RhoB of about 2 h [36],excluding that increased half-live of RhoB protein contributed to TcsL-induced RhoB expression.

3.7. Cytoprotective effect of RhoB

RhoB is regarded as a sensor of cell damage and and a key regulator of either cytoprotective responses [37,38] or of apoptotic cell death [39,7,6]. The role of RhoB in TcsL-induced apoptosis was comparatively analysed in E1A/Ras(ER)-transformed RhoB+/– and RhoB–/– MEFs [25,7]. TcsL-induced RhoB expression was specifically observed in RhoB+/– MEFs (Fig. S7). TcsL concentration-dependently glucosylated (H/K/N)Ras in ER-transformed RhoB+/– and RhoB–/– MEFs (Fig. S6). TcsL-induced activation of the effector caspase-3 was analyzed in terms of the cleavage of its colorimetric substrate DEVD-pNA. TcsL induced pronounced

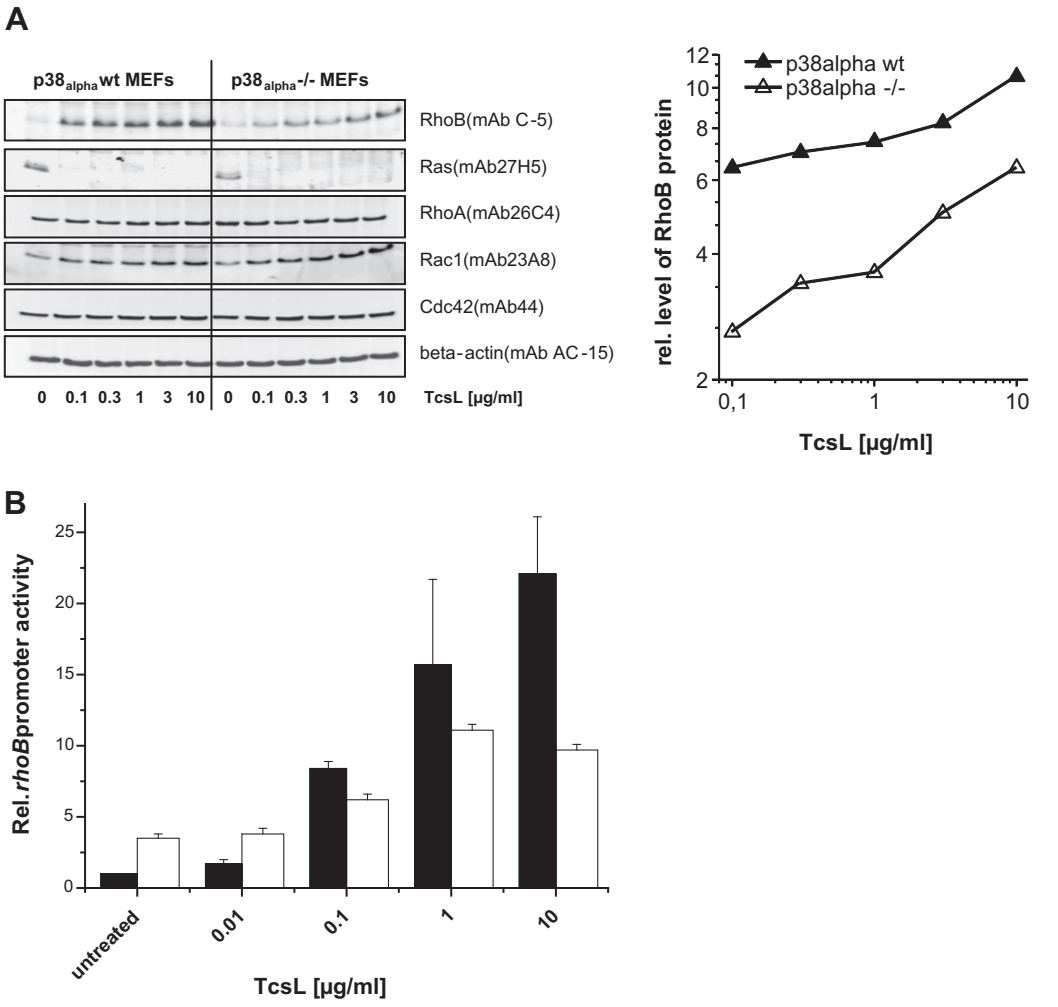

Fig. 5. Role of p38 MAP kinase in TcsL-induced RhoB expression. (A) Role of $p38_{alpha}$ in TcsL-induced RhoB expression. $p38_{alpha}$wt and $p38_{alpha}-/-$ MEFs were exposed to the indicated concentrations of TcsL for 4 h. Western blot analysis was performed with the indicated antibodies. RhoB signals from three independent experiments were analyzed densitometrically and normalized to beta-actin signals. The level of RhoB in untreated cells was set 1.0. Data shown represent the mean of three independent experiments. (B) Role of $p38_{alpha}$ in TcsL-induced *rhoB* promoter activation. $p38_{alpha}$wt (filled bars) and $p38_{alpha}-/-$ (empty bars) MEFs were transfected with the 3.5 kb *rhoB* promoter CAT-construct followed by treatment of the cells with the indicated concentrations of TcsL for 7 h. Cells were harvested for analysis of CAT protein levels using ELISA. Data shown represent the mean (± S.D.) of three independent experiments.

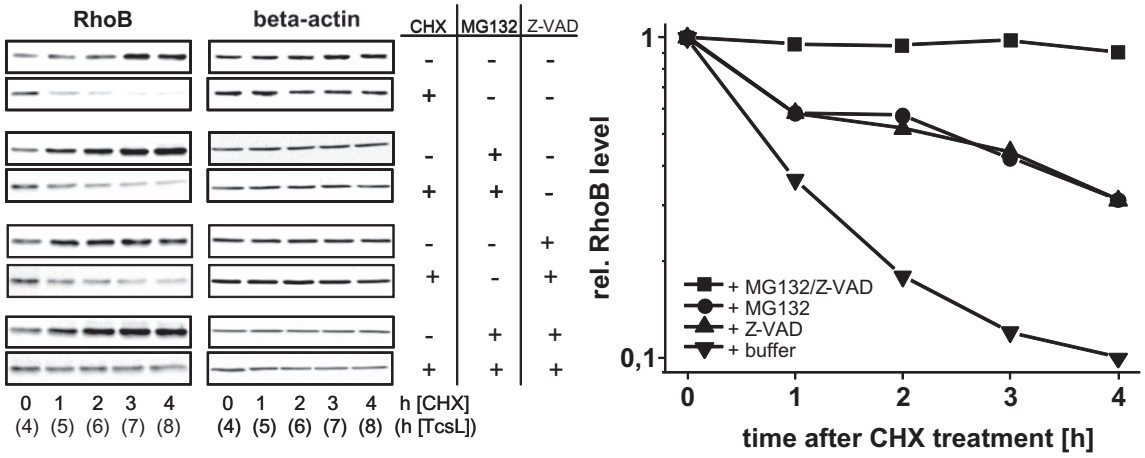

Fig. 6. Proteasome- and caspase-dependent degradation of RhoB. Fibroblasts pre-treated with MG132 (20 µM) or Z-VAD-fmk (20 µM) as indicated for 1 h was exposed to TcsL (3 µg/ml) for 4 h. Subsequently, protein de novo synthesis was blocked by cycloheximide (100 µM) and incubation continued for other 4 h. Cellular RhoB levels were analysed using immuno-blot analysis. Immuno-blots from representative experiments are presented ($n = 3$). Signals were analyzed densitometrically. RhoB signals were normalized to beta-actin signals. Normalized RhoB signal intensity at $t = 0$ was set 1.0. Results displayed are the mean ± S.D. of three independent experiments.

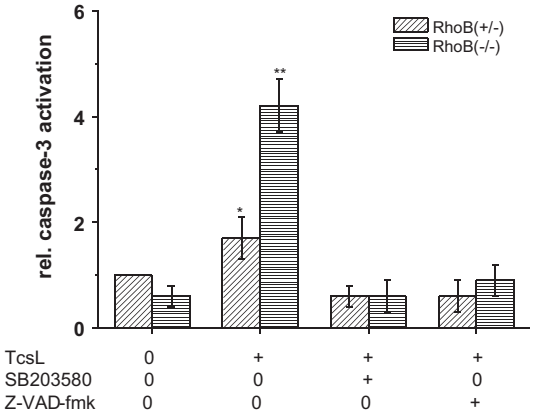

Fig. 7. Protective role of RhoB in TcsL-induced apoptosis. E1A/Ras-transformed RhoB-proficient and RhoB-deficent MEFs treated with either Z-VAD-fmk (20 μM), SB203580 (10 μM), or TcsL (300 ng/ml) as indicated for 8 h. Cells were lysed and the activity of caspase-3 was analyzed using the caspase-3 colorimetric assay. Caspase-3 activity in non-treated cells was set 1.0. Results displayed represent the mean (± S.D.) out of three independent experiments. *P*-values < 0.05 (*) and < 0.01 (**) were considered as statistically significant as compared with non-treated cells.

caspase-3 activation in RhoB-deficient MEFs, while in RhoB proficient MEFs only moderate caspase-3 activation was observed (Fig. 7). This finding suggested that RhoB exhibits a cytoprotective rather than a pro-apoptotic effect in TcsL-induced apoptosis. Both Z-VAD-fmk and SB203580 have been described as efficient inhibitors of TcsL-induced apoptosis in several cell culture models [20,21,35]. In both RhoB+/− and RhoB−/− MEFs, TcsL-induced caspase-3 activation was completely inhibited by either SB203580 or Z-VAD-fmk (Fig. 7), suggesting that the cytoprotective effect of each inhibitor was independent of RhoB. Taken together, the presence of RhoB reduced cytotoxic activity of TcsL, suggesting that RhoB acted in a cytoprotective manner.

4. Discussion

RhoB is the only member of the Rho family of low molecular mass GTP binding proteins that is an immediate early gene product [11]. The cellular level of RhoB is low in cultured cell lines, due to repression of *rhoB* promoter activity by (H/K/N)Ras in a PI3K-dependent manner [8,9]. The observations of the present study on TcsL-induced RhoB expression are fully consistent with the concept that RhoB expression is based on de-repression of Ras/PI3K-dependent RhoB repression: (i) TcsL treatment resulted in de-repression of the rhoB promoter and subsequent RhoB expression most likely based on (H/K/N)Ras glucosylation, (ii) inhibition of PI3K by LY294002 were sufficient to induce moderate RhoB expression, (iii) TUDCA-mediated preservation of PI3K activity (downstream of glucosylated Ras) restored RhoB repression in TcsL-treated fibroblasts, (iv) TcdBF, that shares all substrates of TcsL except for (H/K/N)Ras, did not induce RhoB expression, (v) knock-down of K-Ras was sufficient for RhoB expression in Hela cells suggests that it is specifically K-Ras that mediates RhoB repression. In sum, these observations strongly suggest that TcsL-induced RhoB expression is based on the inhibition of Ras/PI3K signaling. The results of this study do not fully rule out the possibility that TcsL induces RhoB expression in a Ras-independent manner. Analysis of RhoB expression in TcsL-treated cells with Ras knocked-out would provide evidence on the critical role of Ras glucosylation in TcsL-induced RhoB expression.

K-Ras has been identified as the major activator of PI3K/Akt signaling [40–43]. It appears plausible that RhoB is repressed by K-Ras/PI3K/Akt signaling. In a former study, ectopic expression of each H-Ras, K-Ras, and N-Ras repressed the activity of the rhoB promoter as well as the expression of RhoB protein with comparable efficacy [8]. H-Ras, K-Ras, and N-Ras have thereby been highly over-expressed in NIH3T3 fibroblasts [8], experimental conditions that do not allow the differentiation of Ras isoform-specific effects.

TcsL-induced activation of p38 MAP kinase has been reported for several cultured cell lines, including Hela cells [23] and macrophage-like J774A.1 cells [21]. TcsL-induced p38 activation most likely depends on Rac1 glucosylation, as ectopic expression of dominant-negative Rac1-A17N was sufficient for the activation of p38 MAP kinase [44]. This view is supported by the observation that the activation of p38 MAP kinase correlated with Rac1 (not (H/K/N)Ras) glucosylation in TcsL-treated fibroblasts. Rac1 and (H/K/N)Ras glucosylation exhibited distinct kinetics, with Rac1 being glucosylated by about two orders of magnitude less efficient than (H/K/N)Ras. Comparable observations are also reported for TcsL-treated rat basophilic leukemia cells [17].

p38 MAP kinase contributes to gene expression either by stabilizing mRNA [45,46] or by promoter activation [31,47]. The results of this study suggest that $p38_{alpha}$ influences to RhoB expression rather through activation of the *rhoB* promoter than through stabilizing *rhoB*-mRNA. The *rhoB* promoter harbors a distal CCAAT box at −72 and an inverted CCAAT box at −23 respective to the transcriptional initiation site [31,48]. RhoB expression resulting from HDAC inhibition or induced by UV radiation has been suggested to result from the binding of activating transcription factor 2 (ATF2) via NF-Y to the inverted CCAAT box (−23) in a p38-independent manner [49,50]. More recently, the association of c-Jun and p300 proteins with the distal CCAAT box (−72) has been shown to contribute to UV-induced RhoB expression, with the recruitment of c-Jun and p300 proteins depending on p38 MAP kinase [31]. The latter model might be applicable to TcsL-induced RhoB expression as well. A possible involvement of c-Jun and p300 proteins in TcsL-induced activation of the *rhoB* promoter remains to be established. In TcdBF-treated cells, Rac1 glucosylation results in p38 activation as well (data not shown). Neither activation of the *rhoB* promoter (data not shown) nor RhoB expression were observed in TcdBF-treated cells, showing that p38 activation *per se* was not sufficient for RhoB expression. This leads to the conclusion that RhoB expression includes both de-repression of the *rhoB* promoter by Ras inhibition as well as activation of the *rhoB* promoter by $p38_{alpha}$.

TcdBF treatment caused RhoB activation (i.e. GTP binding), although it did not induce RhoB expression. RhoB expression and RhoB activation thus appeared to be distinguishable effects. In TcdBF-treated cells basal RhoB was activated. RhoA and RhoB are identical on amino acid level within the switch regions involved in GEF interaction [51]. Therefore, it is feasible that RhoA and RhoB share their activating GEF proteins. TcdBF treatment has formerly been demonstrated to result in RhoA activation [52]. Those GEF proteins involved in RhoA activation might mediate RhoB activation in TcdBF-treated and TcsL-treated cells as well. In TcsL-treated cells, the level of RhoB-GTP increases (compared to that in TcdBF-treated cells) due to newly expressed RhoB protein.

The half-life of RhoB protein was less than 1 h in TcsL-treated cells, which, to our best knowledge, is the lowest value ever reported. Former reports have referred to an half-life of RhoB protein of approximately 2 h [36,53]. In general, the cellular level of Rho proteins is regulated by a balance of protein de novo synthesis and proteasomal degradation [34,54]. TcsL treatment results in caspase activation [21,20,35,22]. Therefore, the cellular level of RhoB protein is – besides proteasomal degradation – further regulated by caspase-dependent degradation. This observation was unexpected, as Rho family proteins have not been described as caspase substrates before. Caspase-dependent degradation appears to be a specific feature of RhoB, as degradation of other Rho subtype proteins such as RhoA, Rac1, or Cdc42 was not observed within the analysed time frame of 4 h (data not shown). In silico analysis of

caspase-dependent degradation of Rho proteins using the ExPASy PeptideCutter tool reveals that RhoA and RhoB exhibit a single caspase cleavage site at aspartate-45 [55]. The PeptideCutter does not predict such a single caspase cleavage site on Rac1 or Cdc42. Caspase-dependent degradation implies direct interaction of RhoB with caspases. Such direct interaction of caspase-2 with RhoB has been reported in lovastatin-treated cells [56]. RhoB thus represents a feasible substrate of caspases.

Differential analysis of TcsL-induced caspase-3 activation in RhoB-proficient and RhoB-deficient MEFs revealed that caspase-3 activation is suppressed in the presence of RhoB. In TcsL-treated cells, RhoB expression may be interpreted as a protective response in order to counterbalance the cell death promoting effect resulting from inhibition of Ras-regulated pro-survival signaling. RhoB is degraded by caspases, which implies a termination of its cytoprotective activity. Caspase-dependent RhoB degradation may be interpreted as part of an amplification loop aiming to trigger pro-apoptotic signaling and apoptosis execution. The precise link between RhoB and caspase regulation remains to be established. Yet, it is feasible that it involves the RhoB effector protein Rho-kinase, as Rho-kinase regulates caspase activity in Jurkat cells [57]. Alternatively, RhoB-dependent regulation of caspases may involve the effector protein mDia1 [58]. Finally, altered apoptotic responses including changed expression and activation of several caspases have been described in neurons lacking RhoB [59].

Based on their capability of inhibiting oncogenic Ras and causing re-expression of RhoB in transformed cells, FTIs have been investigated for their potential use as anti-cancer drugs [5]. Their anti-cancer efficacy, however, is disappointing [60]. The observations from TcsL-treated cells might offer an explanation for the poor anti-cancer efficacy of FTI. RhoB appears to act in a cytoprotective (rather than a pro-apoptotic) manner in the context of TcsL- or FTI-mediated Ras inhibition.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2012.08.024.

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